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Five new species of *Hymenoscyphus* (*Helotiaceae, Ascomycota*) with notes on the phylogeny of the genus

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Abstract — Five new species of *Hymenoscyphus—H. aurantiacus*, *H. macrodiscus*, *H. qinghaiensis*, *H. tetrasporus*, and *H. yui*—are recognized by morphological characters and DNA sequence data. Detailed descriptions, illustrations, and discussions concerning morphologically similar and phylogenetically closely related species are provided for each species. The phylogeny of 39 *Hymenoscyphus* species was explored based on the individual internal transcribed spacer of nrDNA, D1/D2 domain of 28S nrDNA, and partial β-tubulin gene sequences, as well as the combined dataset. The sampled species clustered as a highly supported group in the phylogenetic trees with six main clades formed. Correlations between the morphological and phylogenetic features are discussed.

Key words — China, *Leotiomycetes*, taxonomy

Introduction

The genus *Hymenoscyphus* was established by Gray (1821) and lectotypified with *H. fructigenus* (Bull.) Gray (Dennis 1964). It is one of the largest genera of *Helotiaceae* and has a cosmopolitan distribution (Kirk et al. 2008). About 155 species were known in the genus according to Kirk et al. (2008) with a few more species added subsequently (Han & Shin 2008; Queloz et al. 2011; Baral & Bemmann 2013; Zheng & Zhuang 2013a,c, 2014; Gross & Han 2015; Gross et al. 2015).

Species of *Hymenoscyphus* are mainly saprotrophic on plant materials such as wood, twigs, fruits, leaves and herbaceous stems, producing small, stipitate to sessile, white- to yellow-colored apothecia and having an ectal excipulum composed of non-gelatinized textura prismatica to textura angularis parallel or at a low angle to the receptacle surface and fusoid, ellipsoid, to scutuloid hyaline ascospores. Morphological characteristics used to identify *Hymenoscyphus*

species include apothecial shape, size, and color; excipular structure; the ascal apical apparatus, basal structure, shape, and size; and shape, size, guttulation, septation, and presence of cilia of ascospores. Habitat is another useful feature for species recognition. Sequence data of the internal transcribed spacer of nuclear ribosomal DNA (ITS) have been introduced to aid identification (Zhang & Zhuang 2004, Baral et al. 2006, Han & Shin 2008, Johnston & Park 2013, Zheng & Zhuang 2013a), with multilocus phylogenetic approaches further applied to establish a clear species concept of the *H. albidus* complex (Queloz et al. 2011, Zheng & Zhuang 2014, Gross & Han 2015, Gross et al. 2015).

A total of 42 *Hymenoscyphus* species have been reported from 28 provinces of China (Teng 1963; Tai 1979; Korf & Zhuang 1985, 1989; Zhuang 1995, 1996, 1999; Wu et al. 1996; Zhuang & Wang 1998; Yu et al. 2000; Zhang & Zhuang 2002a,b, 2004; Zheng & Zhuang 2011, 2013a,b,c, 2014), of which 12 were originally described from China (Zhuang 1996; Zhang & Zhuang 2002a,b, 2004; Zheng & Zhuang 2013a,c, 2014; Liu & Zhuang 2015). In this work, five new species are established based on morphological and molecular data.

Materials & methods

Morphological study

Specimens examined were collected from Hubei, Qinghai, Xinjiang, and Yunnan provinces and deposited in Herbarium Mycologicum, Academiae Sinicae, Beijing, China (HMAS). Colors of fresh apothecia were recorded in field notes. Dry apothecia were rehydrated with distilled water and sectioned longitudinally at a thickness of 8–15 µm using a Yidi YD-1508A freezing microtome (Jinhua, China). Measurements were taken from the longitudinal sections and from squash mounts in lactophenol cotton blue solution using an Olympus BH-2 microscope (Tokyo, Japan). Iodine reactions of ascus apparatuses were tested in Melzer's reagent and Lugol´s solution with or without pretreatment by 3% KOH (Baral 2009). Features of ascus apical ring were recorded according to Baral (1986, 1987). Photographs were taken using a Canon G5 digital camera (Tokyo, Japan) connected to a Zeiss Axioskop 2 Plus microscope (Göttingen, Germany) for anatomical structure (lactophenol cotton blue as mounting medium except for otherwise mentioned) and to a Zeiss Stemi 2000C stereomicroscope (Göttingen, Germany) for gross morphology.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from dry apothecia using the CTAB procedure (White et al. 1990) with some modifications. Primer pairs used for amplification and sequencing included ITS1/ITS4 or ITS5/ITS4 (White et al. 1990) for ITS, LR0R (Moncalvo et al. 1995) and LR5 (Vilgalys & Hester 1990) for D1/D2 domain of 28S nrDNA (LSU) and Bt3-LM/Bt10-LM (Myllys et al. 2001) for partial β-tubulin gene (BTU).

PCR amplifications had a final volume of 25 µl, containing 2.5 µl 10× PCR buffer (including $MgCl_2$), 0.5 µl dNTP (10mM each), 1.25 µl of each primer (10 mM),

Table 1. Sequences of *Hyaloscypha* (*Hya.*) and *Hymenoscyphus* (*H.*) used in the phylogenetic analyses, with voucher specimen information and GenBank accession numbers

Species	Specimen/ strain no.	ITS	LSU	BTU
Hya. aureliella	M234	EU940228	EU940152	FJ477050
Hya. fuckelii	M233	EU940230	EU940154	FJ477052
H. albidoides H.D. Zheng & W.Y. Zhuang	HMAS 264140	KF188722	KJ472226	KF597265
	HMAS 264142	KF188723	KJ472227	KF597267
H. albidus (Gillet) W. Phillips	Lav_01	GU586884	—	—
	90812.1	GU586893	—	—
H. aurantiacus	HMAS 264143	KJ472289	KJ472228	KJ472259
	HMAS 264144	KJ472288	KJ472229	KJ472260
H. berggrenii (Cooke & W. Phillips) Kuntze	ICMP 19614	KC164645	KC164640	—
	ICMP 19615	KC164647	—	—
H. brevicellulus H.D. Zheng & W.Y. Zhuang	HMAS 264018	JX977162	KJ472231	KJ472262
	HMAS 264016	JX977155	KJ472230	KJ472261
H. calyculus	HMAS 264145	KJ472290	KJ472232	KJ472263
	HMAS 264146	KJ472291	KJ472233	KJ472264
H. caudatus	HMAS 82057	AY348576	KJ472234	KJ472265
	HMAS 82060	AY348577	KJ472235	KJ472266
H. crataegi Baral & R. Galán	F156966	DQ431177	—	—
H. dehlii M.P. Sharma	HMAS 264160	KC416307	—	—
H. epiphyllus (Pers.) Rehm ex Kauffman	HMAS 82075	AY348580	KJ472236	KJ472267
	HMAS 82076	AY348581	KJ472237	KJ472268
H. fraxineus (T. Kowalski) Baral et al.	HMAS 264174	KF188724	KJ472248	KF597268
	HMAS 266580	KF188726	KJ472249	KF597269
H. fructigenus	Oth_01	GU586904	—	—
	HMAS 75893	JX977144	—	—
	CBS650.92	GU586933	—	—
	ASM 1061	—	JN673046	—
	M159	—	EU940157	FJ477056
H. fucatus (W. Phillips) Baral & Hengstm.	HMAS 264028	JX977147	—	—
	HMAS 264029	JX977148	—	—
H. ginkgonis J.G. Han & H.D. Shin	KUS-F51352	EU096525	—	—
	KUS-F51854	EU219982	—	—
H. globus W.Y. Zhuang & Yan H. Zhang	HMAS 82107	AY348593	KJ472238	KJ472269
H. haasticus P.R. Johnst.	ICMP 19598	KC164648	—	—
	ICMP 19603	KC164644	KC164639	—
H. himalayensis (K.S. Thind & H. Singh) K.S. Thind & M.P. Sharma	HMAS 188545	KJ472292	—	—
H. hyaloexcipulus	HMAS 188542	JX977145	—	—

Table 1, concluded on p. 1020

Table 1, concluded

Species	Specimen/ strain no.	ITS	LSU	BTU
H. immutabilis	HMAS 71809	AY348584	KJ472239	KJ472270
	F155012	DQ431175	—	—
H. jinggangensis Yan H. Zhang & W.Y. Zhuang	HMAS 82036	KJ472293	KJ472240	KJ472271
	HMAS 188548	KJ472294	KJ472241	—
	HMAS 264156	KJ472295	—	KJ472272
H. kiko P.R. Johnst.	ICMP 19613	KC164656	—	—
	ICMP 19597	KC164661	KC164642	—
H. lasiopodius (Pat.) Dennis	HMAS 71820	AY348585	KJ472242	KJ472273
	HMAS 75878	AY348587	KJ472243	KJ472274
H. macrodiscus	HMAS 264158	KJ472296	—	—
H. macroguttatus	HB7034	DQ431179	—	—
	HMAS 264159	KC416306	KJ472244	KJ472275
H. microcaudatus H.D. Zheng & W.Y. Zhuang	HMAS 264020	JX977156	KJ472245	KJ472276
H. microserotinus (W.Y. Zhuang) W.Y. Zhuang	HMAS 68520	DQ986481	—	—
	HMAS 264030	JX977150	KJ472246	KJ472277
	HMAS 264031	JX977151	KJ472247	KJ472278
H. cf. *monticola*	HMAS 188562	KJ472301	—	—
H. ohakune P.R. Johnst.	ICMP 19601	KC164649	—	—
	ICMP 19607	KC164650	—	—
H. qinghaiensis	HMAS 264175	KJ472297	—	—
H. scutula (Pers.) W. Phillips	HMAS 264178	KC416308	—	KJ472279
	HMAS 264180	KC416309	KJ472250	—
H. scutuloides	HMAS 82092	AY348589	KJ472251	KJ472280
	HMAS 82093	AY348590	KJ472252	KJ472281
H. serotinus	F093261	DQ431168	—	—
	F156526	DQ431178	—	—
H. sinicus	HMAS 71818	KJ472298	—	KJ472282
	HMAS 264193	KJ472299	KJ472253	KJ472283
	HMAS 264194	KJ472300	KJ472254	–
H. subpallescens Dennis	HMAS 264022	JX977154	KJ472255	KJ472284
	HMAS 264026	JX977160	KJ472256	KJ472285
H. subsymmetricus H.D. Zheng & W.Y. Zhuang	HMAS 264021	JX977153	KJ472257	KJ472286
H. tamaricis R. Galán et al.	br020	DQ431167	—	—
	F110125	—	FJ005156	—
H. tetrasporus	HMAS 266592	KJ472302	—	—
H. waikaia P.R. Johnst.	PDD 102886	KC164667	—	—
	ICMP 19599	KC164646	KC164641	—
H. yui	HMAS 266594	KJ472304	—	—
	HMAS 266595	KJ472303	KJ472258	KJ472287

2.5 units of Taq DNA polymerase (HT-biotech Co., Ltd., Beijing, China), and 2–3 µl DNA template. PCR reactions were carried out in an Applied Biosystems 2720 thermocycler (Foster City, CA, USA) under the following conditions: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30–60 s, annealing at 50–53 °C for 30–60 s, and extension 1 min at 72 °C, and by a final extension at 72 °C for 10 min.

PCR products were purified using the AxyPrep™ 96-well DNA Gel Extraction Kit (Corning Life Sciences – Axygen Biotechnology Co., Ltd., Hangzhou, China) and sequenced on an ABI 3730 XL DNA Sequencer (Applied Biosciences, Foster City, CA, USA) with the same primer pairs used for PCR amplification at Beijing Tianyi Huiyuan Bioscience and Technology Inc., China.

Sequence assembly, alignment and phylogenetic analyses

Forward and reverse sequences generated in this study were assembled using BioEdit 7.0.5.3 (Hall 1999) or SeqMan (DNASTAR, Lasergene 7.1.0) to obtain a consensus sequence. Sequences used in the phylogenetic analyses were either generated by this study or retrieved from GenBank (Table 1). *Hyaloscypha aureliella* (Nyl.) Huhtinen and *Hyaloscypha fuckelii* Nannf. were used as outgroup taxa. Sequences were aligned with either BioEdit 7.0.5.3 or Muscle 3.8.31 (Edgar 2004), manually edited when needed, and converted to nexus files in ClustalX 1.83 (Thompson et al. 1997).

Phylogenetic analyses were based on individual ITS, LSU and BTU sequences as well as combined dataset. All characters were treated as unordered and equally weighted, and gaps were treated as missing data. Prior to combined analyses, partition homogeneity tests (PHT) were performed in PAUP* 4.0b10 (Swofford 2003) with 100 replicates to confirm the combination of different gene sequences for phylogenetic analyses. PAUP*4.0b10 for Windows (Swofford 2003) was used to conduct neighbor-joining (NJ) and maximum parsimony (MP) analyses. NJ analysis was performed using default settings. MP analysis was done with the heuristic search option using max trees set to 1000 and auto-increased by 100, TBR branch swapping. Tree scores, including tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for all the trees generated. The topological confidence of the resulting trees was tested with bootstrap analysis using 1000 replications, each with 10 replicates of random addition of taxa in both NJ and MP analyses.

Bayesian inference (BI) analysis was performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) under the best-fit evolutionary model estimated by MrModeltest 2.3 with the Akaike information criterion (Nylander 2004). Two parallel runs of four simultaneous chains of Markov Chain Monte Carlo were performed starting from random trees for 2,000,000 generations with the trees sampled every 100 generations (resulting in 20,000 total trees). The first quarter of sampled trees were discarded as the burn-in phase of the analyses, and the remaining trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. The trees were viewed in TreeView 1.6.6 (Page 1996).

Results

ITS, LSU, and BTU sequences of *Hymenoscyphus* were analyzed separately as well as concatenated. The P value of PHT is 0.06 for the combined ITS, LSU,

and BTU matrix, which indicates no significant incongruence among the three markers. For Bayesian analysis, GTR+I+G was selected by MrModeltest as the best-fit model for all the four datasets. The characteristics of each sequence dataset are summarized in Table 2.

Table 2. Sequence characteristics of ITS, LSU, BTU, and combined datasets.

Parameter	ITS	LSU	BTU	Combined dataset
Number of *Hymenoscyphus* sequences (newly generated)	70 (18)	40 (33)	34 (29)	30 (30)
Number of *Hymenoscyphus* species / represented by holotype (newly generated / new species + known species)	39 / 19 (9 / 5+4)	26 / 8 (20 / 2+18)	21 / 9 (20 / 2+18)	19 / 8 (19 / 2+17)
Total nucleotides	593	844	804	2193
Constant characters	345	720	512	1592
Variable but parsimony uninformative characters	44	16	26	87
Parsimony informative characters	204	108	266	514
Tree length (TL)	966	250	950	1622
Consistency index (CI)	0.424	0.620	0.457	0.533
Retention index (RI)	0.788	0.846	0.685	0.740
Rescaled consistency index (RC)	0.334	0.524	0.313	0.394
Homoplasy index (HI)	0.576	0.380	0.543	0.467
Best-fit model	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G

The NJ, MP, and BI analyses produced similar tree topologies except for minor differences in the arrangement of a few terminal branches. The phylograms inferred from NJ analysis of each dataset are shown in Figs 1–3 and statistical supporting values higher than 50% or 0.50 are shown at the nodes. In the phylogenetic trees inferred from all datasets, the sampled species of *Hymenoscyphus* formed a strongly supported group.

The ITS alignment included 70 sequences representing 39 *Hymenoscyphus* species with 593 nucleotides. The ITS barcodes recognized all five putative new taxa that appeared as independent lineages, which give strong support to the morphological observations. In the ITS tree (Fig. 1), the genus were divided into six main clades (A, B, C, D, E, and F) with high statistic supports and a few scattered species. The LSU dataset consisted of 40 samples from 26 species (including two new species) with 844 characters. The LSU tree (Fig. 2A) discriminated the clades A, D, E, and F, while Clade B split into two parts, one together with Clade A and the other with species of Clade C. The BTU matrix comprised 34 sequences from 21 species (including two new species)

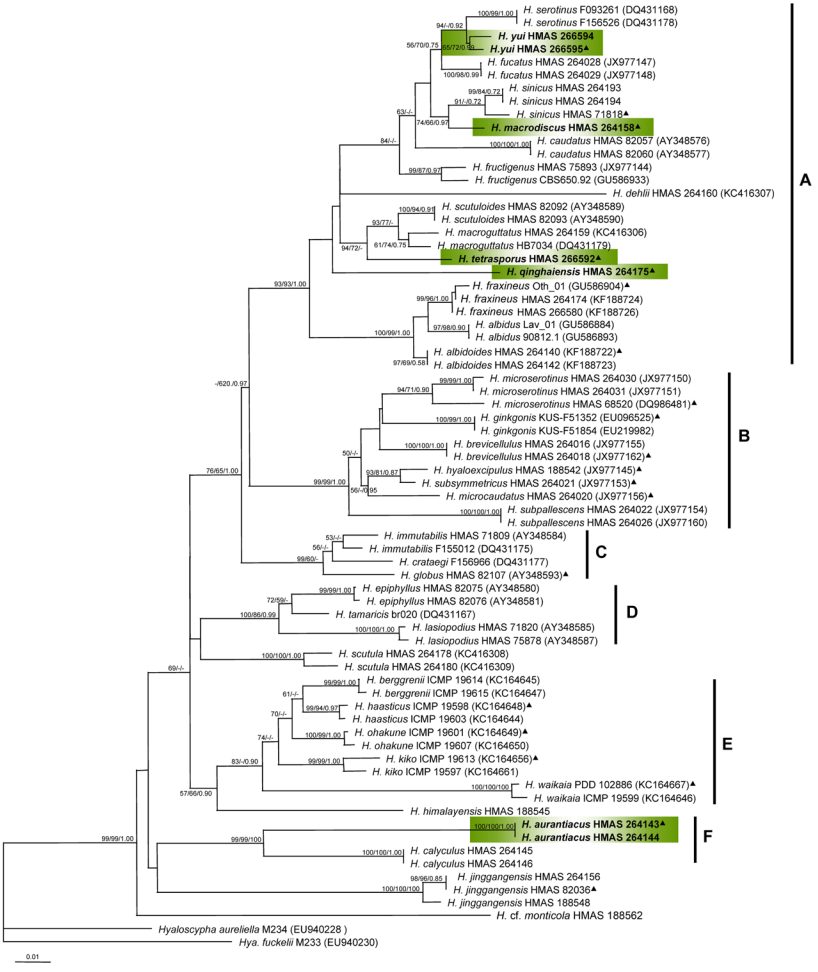
H. serotinus F093261 (DQ431168)
H. serotinus F156526 (DQ431178)
H. yui HMAS 266594
H.yui HMAS 266595
H. fucatus HMAS 264028 (JX977147)
H. fucatus HMAS 264029 (JX977148)
H. sinicus HMAS 264193
H. sinicus HMAS 264194
H. sinicus HMAS 718184
H. macrodiscus HMAS 264158
H. caudatus HMAS 82057 (AY348576)
H. caudatus HMAS 82060 (AY348577)
H. fructigenus HMAS 75893 (JX977144)
H. fructigenus CBS650.92 (GU586933)
H. dehlii HMAS 264160 (KC416307)
H. scutuloides HMAS 82092 (AY348589)
H. scutuloides HMAS 82093 (AY348590)
H. macroguttatus HMAS 264159 (KC416306)
H. macroguttatus HB7034 (DQ431179)
H. tetrasporus HMAS 266592
H. qinghaiensis HMAS 264175
H. fraxineus Oth_01 (GU586904)
H. fraxineus HMAS 264174 (KF188724)
H. fraxineus HMAS 266580 (KF188726)
H. albidus Lav_01 (GU586884)
H. albidus 90812.1 (GU586893)
H. albidoides HMAS 264140 (KF188722)
H. albidoides HMAS 264142 (KF188723)
H. microserotinus HMAS 264030 (JX977150)
H. microserotinus HMAS 264031 (JX977151)
H. microserotinus HMAS 68520 (DQ986481)
H. ginkgonis KUS-F51352 (EU096525)
H. ginkgonis KUS-F51854 (EU219982)
H. brevicellulus HMAS 264016 (JX977155)
H. brevicellulus HMAS 264018 (JX977162)
H. hyaloexcipulus HMAS 188542 (JX977145)
H. subsymmetricus HMAS 264021 (JX977153)
H. microcaudatus HMAS 264020 (JX977156)
H. subpallescens HMAS 264022 (JX977154)
H. subpallescens HMAS 264026 (JX977160)
H. immutabilis HMAS 71809 (AY348584)
H. immutabilis F155012 (DQ431175)
H. crataegi F156966 (DQ431177)
H. globus HMAS 82107 (AY348593)
H. epiphyllus HMAS 82075 (AY348580)
H. epiphyllus HMAS 82076 (AY348581)
H. tamaricis br020 (DQ431167)
H. lasiopodius HMAS 71820 (AY348585)
H. lasiopodius HMAS 75878 (AY348587)
H. scutula HMAS 264178 (KC416308)
H. scutula HMAS 264180 (KC416309)
H. berggrenii ICMP 19614 (KC164645)
H. berggrenii ICMP 19615 (KC164647)
H. haasticus ICMP 19598 (KC164648)
H. haasticus ICMP 19603 (KC164644)
H. ohakune ICMP 19601 (KC164649)
H. ohakune ICMP 19607 (KC164650)
H. kiko ICMP 19613 (KC164656)
H. kiko ICMP 19597 (KC164661)
H. waikaia PDD 102886 (KC164667)
H. waikaia ICMP 19599 (KC164646)
H. himalayensis HMAS 188545
H. aurantiacus HMAS 264143
H. aurantiacus HMAS 264144
H. calyculus HMAS 264145
H. calyculus HMAS 264146
H. jinggangensis HMAS 264156
H. jinggangensis HMAS 82036
H. jinggangensis HMAS 188548
H. cf. monticola HMAS 188562
Hyaloscypha aureliella M234 (EU940228)
Hya. fuckelii M233 (EU940230)
A
B
C
D
E
F
0.01

Fig. 1. Neighbor-joining tree of selected *Hymenoscyphus* species inferred from ITS sequences. Bootstrap values of NJ (left) and MP (middle) > 50%, and Bayesian posterior probabilities values (right) >0.50 are indicated at the nodes. ▲ indicates an ex-holotype sequence.

containing 804 positions. The clades A, B, C, D, and F were well reconstructed in the tree inferred from BTU with Clade E missing due to unavailability of sequence data (Fig. 2b). The combined three-marker alignment consisted of 30 samples representing 19 species and 2,193 characters including two new species. In the tree generated from the combined dataset (Fig. 3), the clades A, B, C, D, and F were well recognized.

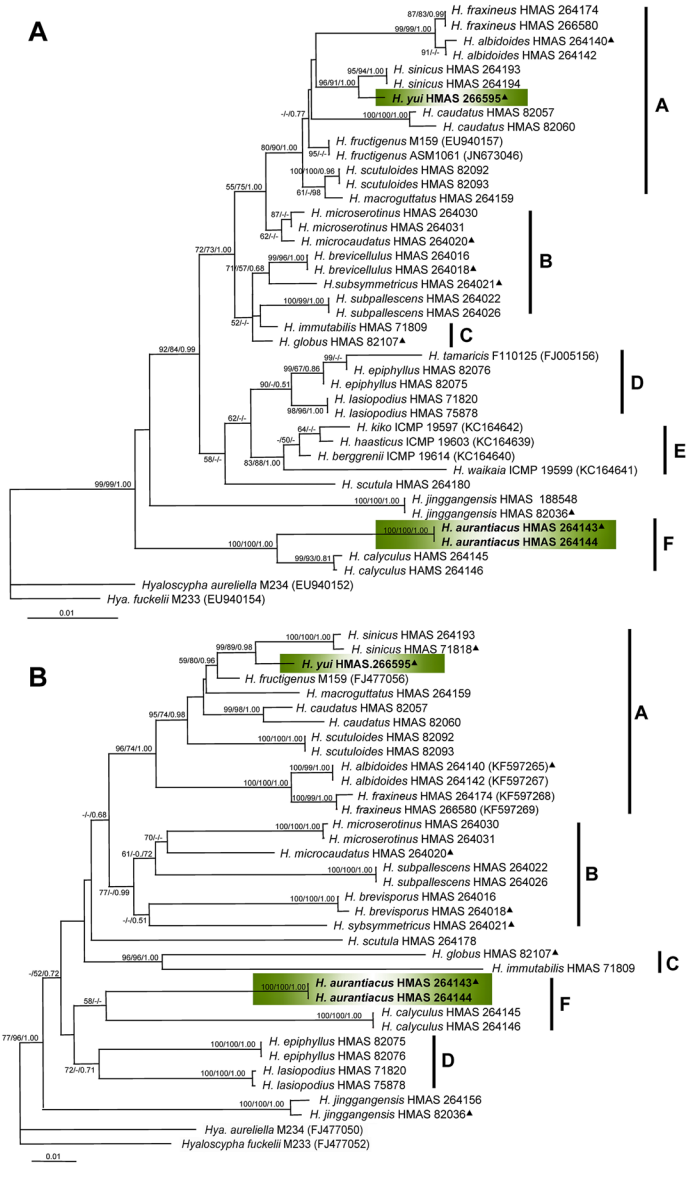

Fig. 2. Neighbor-joining trees of selected *Hymenoscyphus* species inferred from LSU (A) and BTU (B) sequences. Bootstrap values of NJ (left) and MP (middle) > 50%, and Bayesian posterior probabilities values (right) >0.50 are indicated at the nodes. ▲ indicates an ex-holotype sequence.

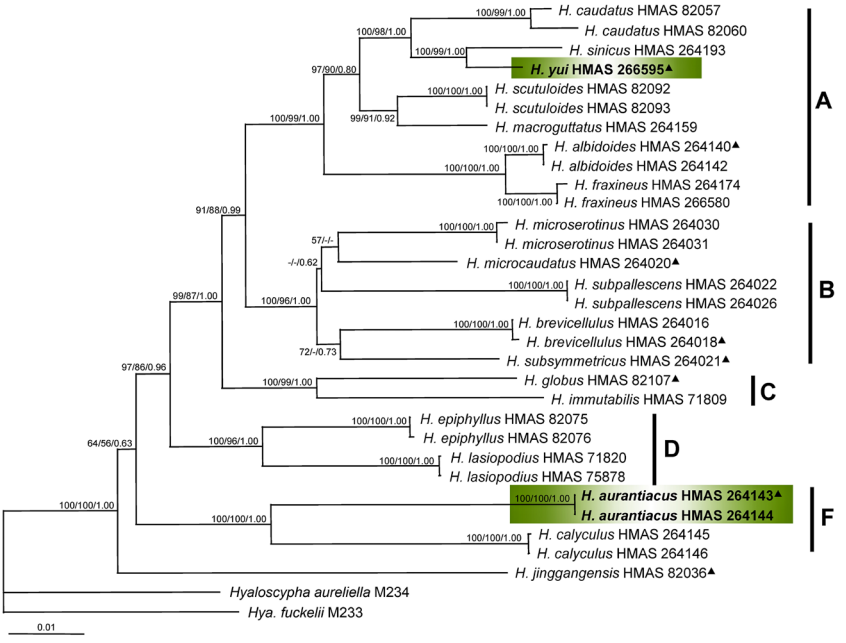

Fig. 3. Neighbor-joining tree of selected *Hymenoscyphus* species inferred from combined ITS, LSU and BTU sequences. Bootstrap values of NJ (left) and MP (middle) > 50%, and Bayesian posterior probabilities values (right) >0.50 are indicated at the nodes. ▲ indicates an ex-holotype sequence.

Taxonomy

Fig. 4. Gross morphology of apothecia (A, C, E, G, I: Rehydrated apothecium; B, D, F, H, J: Dried apothecia on natural substrates). A, B. *Hymenoscyphus aurantiacus* (HMAS 264143, holotype); C, D. *H. macrodiscus* (HMAS 264158, holotype); E, F. *H. qinghaiensis* (HMAS 264175, holotype); G, H. *H. tetrasporus* (HAS 264195, holotype); I, J: *H. yui* (HMAS 266595, holotype). Scale bars: A, B, E, F, G, H = 0.5 mm; C, D, I, J = 1 mm.

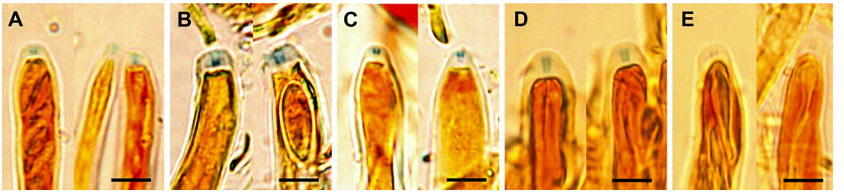

Fig. 5. IKI reaction of ascus apical ring (mounted in Lugol's solution without KOH pretreatment). A. *Hymenoscyphus aurantiacus* (holotype); B. *H. macrodiscus* (holotype); C. *H. qinghaiensis* (holotype); D. *H. tetrasporus* (holotype); E. *H. yui* (holotype). Scale bars = 5 μm.

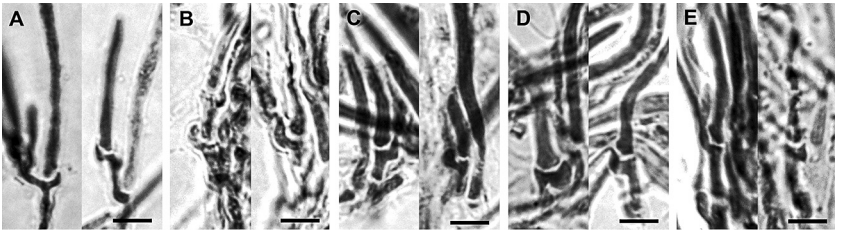

Fig. 6. Ascus bases showing croziers. A. *Hymenoscyphus aurantiacus* (holotype); B. *H. macrodiscus* (holotype); C. *H. qinghaiensis* (holotype); D. *H. tetrasporus* (holotype); E: *H. yui* (holotype). Scale bars = 5 μm.

Hymenoscyphus aurantiacus H.D. Zheng & W.Y. Zhuang, **sp. nov.** Figs 4a,b, 5a, 6a, 7

Fungal Name FN570094

Differs from *Hymenoscyphus imberbis* by its orange, distinctly stipitate apothecia and narrower excipular hyphae.

Type: China. Hubei, Wufeng, Houhe, alt. 800 m, on rotten twig and mossy wood of an unidentified deciduous tree, 12 Nov. 2004, W.Y. Zhuang & C.Y. Liu 5501 (**Holotype,** HMAS 264143).

Gene sequences ex-holotype: KJ472289 (ITS), KJ472228 (LSU), KJ472259 (BTU).

Etymology: The specific epithet refers to the color of the apothecia.

Apothecia scattered or 2–3 in a cluster, discoid, stipitate, 0.8–3 mm in diam.; hymenium surface orange, drying pale brownish to pale orange; receptacle surface paler, drying cream to cream-orange; stipe concolorous with receptacle, glabrous or nearly so, with white to orange-red mycelium at base, 0.5–1.5 mm long. Outer covering layer absent or with loosely interwoven hyphae at stipe surface, hyphae cylindrical, ends obtuse. Ectal excipulum of textura porrecta, 20–50 μm thick, cells hyaline, 10–35 × 2–6 μm. Medullary excipulum of two layers, outer layer of textura porrecta, 20–150 μm thick; inner layer of textura intricata, 30–180 μm thick; hyphae hyaline, 2–4 μm wide. Subhymenium not distinguishable. Hymenium 85–95 μm thick. Asci arising from croziers, clavate, with apex broadly subconical to rounded, 8-spored, J+, apical ring

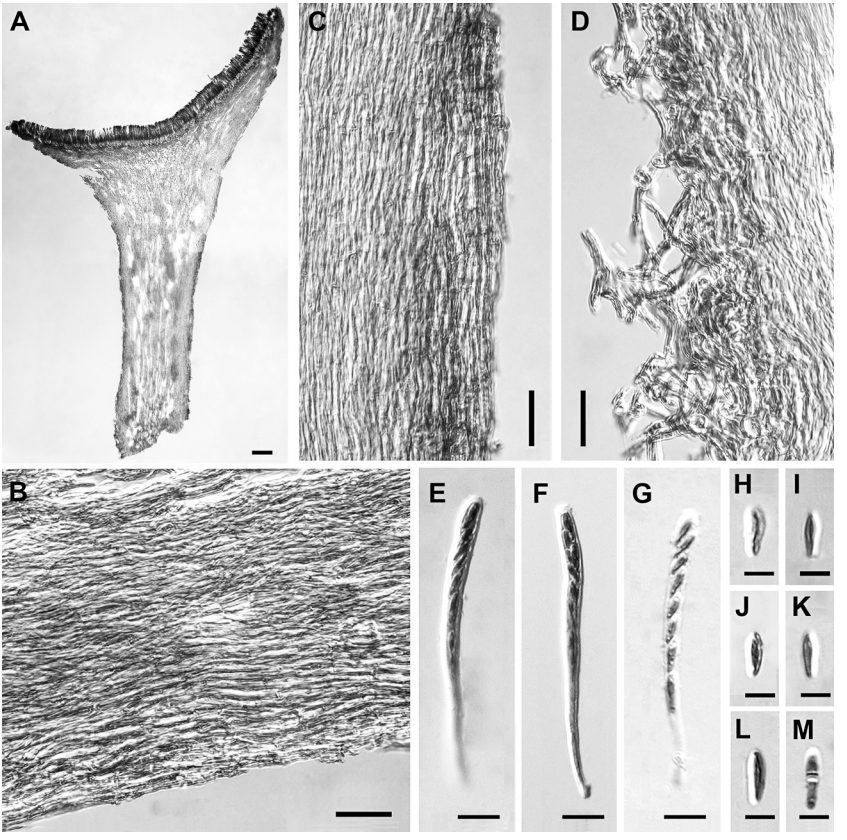

Fig. 7. *Hymenoscyphus aurantiacus* (A–C, E, F, H–K: HMAS 264143, holotype; D, G, L, M: HMAS 264144). A. Longitudinal section of apothecium; B. Excipular structure at flank; C, D. Structure at the middle of stipe; E–G. Ascus with ascospores; H–L. Ascospore; M. 1-septate ascospore. Scale bars: A = 100 μm; B–D = 20 μm; E–G = 10 μm; H–M = 5 μm.

as two blue lines without KOH pretreatment, *Hymenoscyphus*-type, 65–80 × 4.5–5.5 μm. Ascospores clavate to slipper-shaped, upper part slightly enlarged, 0(–1)-septate, hyaline, eguttulate to occasionally with tiny guttules, uniseriate to biseriate above and uniseriate below, 7.8–11 × 2.8–3.3 μm. Paraphyses filiform, hyaline, 1–2 μm wide, equal to or slightly exceeding the asci.

Additional specimen examined — **China. Hubei,** Wufeng, Houhe, alt. 800 m, on rotten seeds of an unidentified plant, 12 Nov. 2004, W.Y. Zhuang & C.Y. Liu 5492 (HMAS 264144).

Notes—In the phylogenetic trees (Figs 1–3), *H. aurantiacus* clustered with *H. calyculus* (Fr.) W. Phillips (Dennis 1956) in the same clade. *Hymenoscyphus*

calyculus differs in its yellow apothecia, broader ectal excipular cells (≤10–25 × 4–12 µm), and cylindrical, biguttulate and much larger ascospores (15–22 × 3–4.5 µm) irregularly biseriate in much larger asci (120–125 × 9–10 µm; Dennis 1956).

Among other *Hymenoscyphus* species, *H. imberbis* (Bull.) Dennis resembles *H. aurantiacus* in asci and ascospores but differs in having pure white apothecia, a short and stout stipe, and broader ectal excipular cells (10–20 × 5–10 µm; Dennis 1956). The new species also resembles *H. leucopsis* (Berk. & M.A. Curtis) Dennis in stipitate apothecia and ectal excipulum structure, but *H. leucopsis* has larger apothecia (4–7 mm diam.), a pallid to copper-colored hymenium surface, and smaller asci (38–52 × 4–5 µm) and ascospores (4–9 × 1.5–2.5; Dumont 1981).

The two *H. aurantiacus* specimens share similar macroscopic and microscopic characteristics as well as identical sequence data. A minor difference observed is that the stipe surface is glabrous in the holotype (Fig. 7c) but hairy in the other collection (Fig. 7d). We treat this as infraspecific variation.

Hymenoscyphus macrodiscus H.D. Zheng & W.Y. Zhuang, **sp. nov.** Figs 4c,d, 5b, 6b, 8

Fungal Name FN570095

Differs from *Hymenoscyphus sinicus* by its parallel outer covering layer, smaller asci, and equilaterally elliptical-fusoid smaller ascospores uniseriate in the asci.

Type: China. Xinjiang, Guozigou, alt. 1400 m, on rotten wood of an unidentified deciduous tree, 11 Aug. 2003, W.Y. Zhuang & Y. Nong 4860 (**Holotype,** HMAS 264158).

Gene sequence ex-holotype: KJ472296 (ITS).

Etymology: The specific epithet refers to the large-sized apothecia.

Apothecia scattered, discoid, stipitate, 3–8 mm in diam.; hymenium surface yellow, drying ochraceous; receptacle surface paler, drying pale ochraceous; stipe slightly rough, not darkening at base, 1–8 mm long. Outer covering layer of textura intricata, up to 35 µm thick, hyphae 2–3 µm wide. Ectal excipulum of textura prismatica, tissues somewhat gelatinized, 8–30 µm thick, cells hyaline, 5–8 µm wide. Medullary excipulum of two layers, outer layer of textura porrecta, 50–110 µm thick; inner layer of textura intricata, 50–200 µm thick; hyphae hyaline, 2–3 µm wide. Subhymenium not distinguishable. Hymenium about 150 µm thick. Asci arising from croziers, subcylindrical, tapered towards base, apex subconical to subtruncate, 8-spored, J+, apical ring as two blue lines without KOH pretreatment, *Hymenoscyphus*-type, 112–150 × 6–7.5 µm. Ascospores elliptical-fusoid, pointed at both ends, hyaline, eguttulate or rarely with very tiny oil drops, obliquely uniseriate, 11–14.5 × 3.3–4.4 µm. Paraphyses filiform, hyaline, 2–3 µm wide, not exceeding the asci.

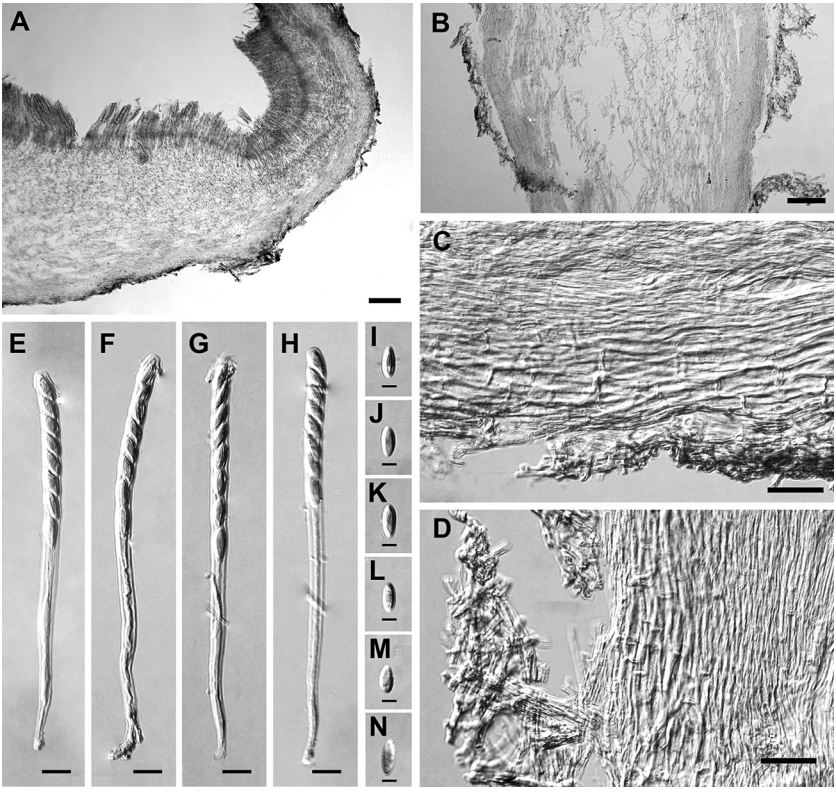

Fig. 8. *Hymenoscyphus macrodiscus* (HMAS 264158, holotype). A. Longitudinal section of portion of apothecium; B. Structure at the middle of stipe; C. Excipular structure at flank; D. Excipular structure at the middle of stipe; E–H. Ascus with ascospores; I–N. Ascospore. Scale bars: A, B = 100 μm; C, D = 20 μm; E–H = 10 μm; I–N = 5 μm.

Notes—In the ITS phylogenetic tree (Fig. 1), *H. macrodiscus* grouped together with *H. sinicus* W.Y. Zhuang & Yan H. Zhang, which is similar in apothecial gross morphology, excipular structure, and habitat but differs in its outer covering layer composed of parallel hyphae, larger asci (125–175 × 8–12.5 μm), and larger ascospores (16–26 × 4.1–6 μm) that are inequilaterally fusoid, guttulate, and occasionally with one or two septa (Zhang & Zhuang 2002b). *Hymenoscyphus immutabilis* (Fuckel) Dennis has similar ascospores, but differs in having white and smaller apothecia (0.8–1.5 mm) with a short stipe, angular cells in ectal excipulum, absence of an outer covering layer, shorter and broader asci (80–105 × 8–10 μm), and foliicolous habitat (White 1943).

Hymenoscyphus qinghaiensis H.D. Zheng & W.Y. Zhuang, **sp. nov.** Figs 4e,f, 5c, 6c, 9

Fungal Name FN570096

Differs from *Hymenoscyphus phyllogenus* by its larger apothecia, broader ectal excipular hyphae, narrower asci arising from croziers, and narrower ascospores.

Type: China. Qinghai, Minhe Xigou, alt. 2600 m, on rotten leaves of *Populus* sp., 10 Aug. 2004, W.Y. Zhuang & C.Y. Liu 5248 (**Holotype,** HMAS 264175).

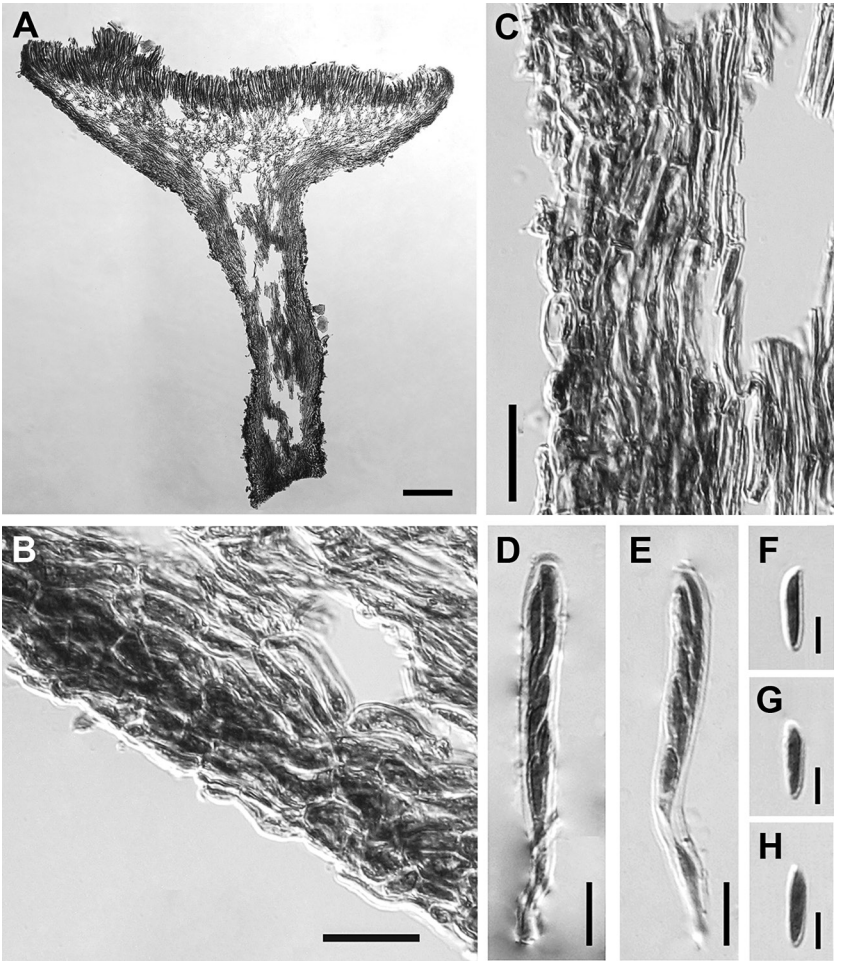

Fig. 9. *Hymenoscyphus qinghaiensis* (HMAS 264175, holotype). A. Longitudinal section of apothecium; B. Excipular structure at flank; C. Excipular structure at the middle of stipe; D, E. Ascus with ascospores; F–H. Ascospore. Scale bars: A = 100 µm; B, C = 20 µm; D, E = 10 µm; F–H = 5 µm.

Gene sequence ex-holotype: KJ472297 (ITS).

Etymology: The specific epithet refers to the type locality of the fungus.

Apothecia scattered, discoid to flat, stipitate, 0.4–1.4 mm in diam.; hymenium surface cream to very pale yellow, drying pale orange; receptacle surface drying cream; stipe concolorous with receptacle surface, glabrous or nearly so, not dark at base, 0.2–0.7 mm long. Outer covering layer of 1–3 hyphal layers, hyphae about 3 µm wide. Ectal excipulum of textura prismatica, 15–60 µm thick, cells hyaline, 8–30 × 5–10 µm. Medullary excipulum of two layers, outer layer of textura porrecta, 10–55 µm thick; inner layer of textura intricata, 30–140 µm thick; hyphae hyaline, 2–4 µm wide. Subhymenium not distinguishable. Hymenium 80–85 µm thick. Asci arising from croziers, clavate, apex obtuse to conical, 8-spored, J+, apical ring as two blue lines without KOH pretreatment, *Hymenoscyphus*-type, 68–89 × 6.7–7.8 µm. Ascospores cylindric-ellipsoid-fusoid, posterior end pointed, anterior end partly asymmetrical, flattened on one side, hyaline, eguttulate or rarely with very small guttules, uniseriate to biseriate above and uniseriate below, 11–14.5 × 3.3–4 µm. Paraphyses filiform, hyaline, 1.5–2.5 µm wide, not exceeding the asci.

Additional specimens examined — **CHINA. Qinghai,** Minhe Xigou, alt. 2600 m, on rotten leaves of *Populus* sp., 10 Aug. 2004, W.Y. Zhuang & C.Y. Liu 5241, 5247 (HMAS 264176, 264177).

Notes—Among the known *Hymenoscyphus* species growing on rotten *Populus* leaves having similar asci and ascospores, *H. phyllogenus* (Rehm) Kuntze is most similar to *H. qinghaiensis* but differs in having much smaller apothecia (0.3–0.5 mm diam.), narrower ectal excipular cells (15–20 × 4–5 µm wide), broader asci (65–75 × 8.5–11 µm) arising from simple septa, and broader ascospores (11–14 × 3.8–5 µm; White 1943; Dennis 1956). The new species is also similar to *H. albopunctus* (Peck) Kuntze, which differs obviously in broader asci (60–70 × 8–10 µm) arising from simple septa, ascospores with 2–4 conspicuous oil drops, and growing on leaves of *Fagus* sp. (White 1943).

In the ITS phylogenetic tree (Fig. 1), *H. qinghaiensis* stands in a distinct lineage within Clade A and of uncertain relationship with other species.

Hymenoscyphus tetrasporus H.D. Zheng & W.Y. Zhuang, **sp. nov.** Figs 4g,h, 5d, 6d, 10

Fungal Name FN570098

Differs from all the known *Hymenoscyphus* species by its asci containing 4 ascospores at maturity.

Type: China. Hebei, Wulingshan Lianhuachi, alt. 1800 m, on herbaceous stem of an unidentified monocot, 26 Aug. 1989, W.Y. Zhuang 490 (**Holotype,** HMAS 266592).

Gene sequence ex-holotype: KJ472302 (ITS).

Etymology: The specific epithet refers to the number of ascospores in mature asci.

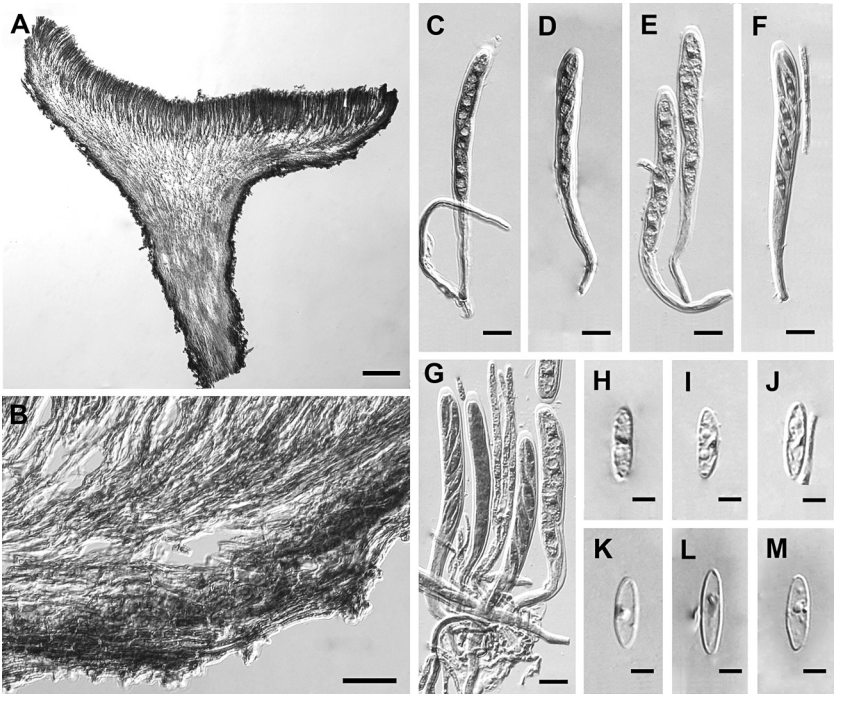

Fig. 10. *Hymenoscyphus tetrasporus* (HMAS 264195, holotype). A. Longitudinal section of apothecium; B: Excipular structure at flank; C–E: Mature ascus / asci with four ascospores; F: Immature ascus with four normal ascospores and four degenerated ones; G: Asci at different developmental stages; H–M: Ascospore. Scale bars: A = 100 μm; B = 20 μm; C–G = 10 μm; H–M = 5 μm.

Apothecia scattered, discoid, stipitate, 1–2 mm in diam.; hymenium surface yellow, drying pale brownish yellow; receptacle surface drying pallid to light orange; stipe concolorous with receptacle surface, glabrous or nearly so, 0.5–1.2 mm long. Outer covering layer about 15 μm thick, hyphae with walls roughened, about 2 μm wide. Ectal excipulum of textura prismatica, glabrous or with short hyphal protrusions, 20–50 μm thick, cells hyaline, 6–15 × 4–8 μm. Medullary excipulum of two layers, outer layer of textura porrecta, 30–40 μm thick, inner layer of textura intricata, 50–90 μm thick; hyphae hyaline, 2–3 μm wide. Subhymenium not distinguishable. Hymenium about 125 μm thick. Asci arising from croziers, subcylindrical to clavate, apex obtuse, 8-spored when young, with 4 mature spores at maturity, J+, apical ring as two blue lines without KOH pretreatment, *Hymenoscyphus*-type, 100–115 × 7–9 μm. Ascospores elliptical-fusoid with blunt ends, hyaline, having a conspicuous central dark stained area in cotton blue, with 2–4 large oil drops and several

small ones, uniseriate, (16–)17.8–22 × 4.5–5.5 µm. Paraphyses filiform, 2.5–4 µm wide, equal to or slightly exceeding the asci.

Notes—The most distinctive character of *H. tetrasporus* is that four of the eight ascospores present in young asci abort during development. Therefore, each mature ascus contains only four ascospores (Fig. 10c–g). This prominent character easily distinguishes it from its morphologically most similar species, *H. caudatus* (P. Karst.) Dennis and *H. hyaloexcipulus* H.D. Zheng & W.Y. Zhuang.

Phylogenetically (Fig. 1) this new species grouped together with *H. macroguttatus* Baral et al. and *H. scutuloides* Hengstm., which are similar in anatomic structure, the asci arising from croziers, and guttulate ascospores. *Hymenoscyphus macroguttatus* differs in 8-spored, shorter and wider asci (77–97 × 9.5–11 µm) and shorter (13.5–18 × 4–5.5 µm) and scutuloid ascospores (Zheng & Zhuang 2013b). *Hymenoscyphus scutuloides* is distinguished by 8-spored asci and scutuloid ascospores with cilia at one or both ends (Hengstmengel 1996).

Hymenoscyphus yui H.D. Zheng & W.Y. Zhuang, **sp. nov.** Figs 4i,j, 5e, 6e, 11

Fungal Name FN570099

Differs from *H. macrodiscus* by its outer covering layer of parallel hyphae, ectal excipular hyphae slightly undulate, shorter asci, and inequilaterally elongate-ellipsoid ascospores.

Type: China, Xinjiang, Guozigou, alt. 1800 m, on rotten twig of unidentified deciduous tree, 11 Aug. 2003, W.Y. Zhuang & Y. Nong 4877 (**Holotype,** HMAS 266595).

Gene sequences ex-holotype: KJ472303 (ITS), KJ472258 (LSU), KJ472287 (BTU).

Etymology: The specific epithet honors the late distinguished Chinese mycologist, Prof. Y.N. Yu.

Apothecia scattered, discoid, stipitate, 2–4 mm in diam.; hymenium surface yellow, drying dull orange to brownish orange; receptacle surface paler, drying cream-orange; stipe concolorous with receptacle surface, glabrous or nearly so, having whitish mycelium at base, 0.5–8 mm long. Outer covering layer of 2 or more hyphal layers, hyphae 2–3 µm wide. Ectal excipulum of textura prismatica, 40–70 µm thick, hyphae slightly undulated and somewhat gelatinized, cells hyaline, 10–25 × 3–7 µm. Medullary excipulum of two layers, outer layer of textura porrecta, 20–80 µm thick; inner layer of textura intricata, 40–150 µm thick; hyphae hyaline, 2–3 µm wide. Subhymenium not distinguishable. Hymenium 110–125 µm thick. Asci arising from croziers, subcylindrical, apex rounded, 8-spored, J+, apical ring as two blue lines without KOH pretreatment, *Hymenoscyphus*-type, 86–115 × 5.5–8.5 µm. Ascospores elongate ellipsoid, slightly flattened on one side, hyaline, eguttulate to occasionally with a few tiny oil drops, uniseriate, 11–15.5 × 3.3–5 µm. Paraphyses filiform, hyaline, 1.5–2.5 µm wide, not exceeding the asci.

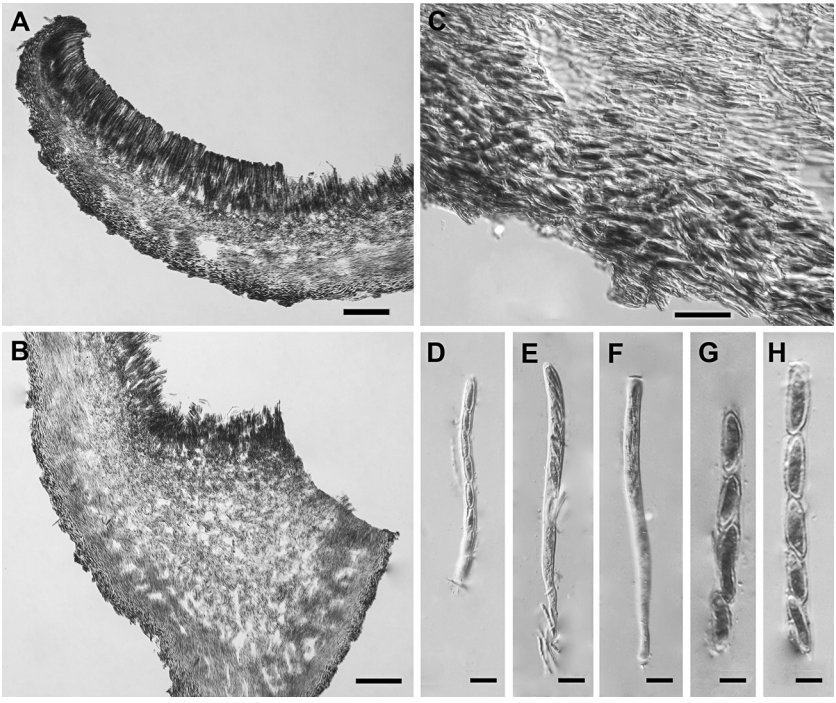

Fig. 11. *Hymenoscyphus yui* (HMAS 266594). A, B. Longitudinal section of portion of apothecium; C. Excipular structure at flank; D–F. Immature ascus; G, H. Ascospores in ascus. Scale bars: A, B = 100 μm; C = 20 μm; D–F = 10 μm; G, H = 5 μm.

Additional specimen examined — **CHINA. Xinjiang,** Yining, Qabqal, alt. 2000 m, on rotten wood of an unidentified deciduous tree, 13 Aug. 2003, W.Y. Zhuang & Y. Nong 4911 (HMAS 266594).

Notes — In the ITS tree (Fig. 1), *H. yui* appears to be a sister of *H. serotinus* (Pers.) W. Phillips, which is quite different in ectal excipulum structure, and bigger (18–28 × 3–4 μm), guttulate, strongly heteropolar and curved ascospores biseriate in asci (Dennis 1956; Baral & Bemmann 2013). In the trees inferred from LSU, BTU, and multilocus data (Figs 2,3), the new species clusters with *H. sinicus* in a highly supported subclade. *Hymenoscyphus sinicus* is similar in apothecial size and color, excipulum structure, and lignicolous habitat but differs in larger asci (125–175 × 8–12.5 μm) and guttulate ascospores, which are larger (16–26 × 4.1–6 μm; Zhang & Zhuang 2002b).

The apothecial gross morphology and habitat of *H. yui* are also similar to *H. macrodiscus*, which differs by its interwoven hyphae in the outer covering layer, straight instead of undulate hyphae in ectal excipulum, longer asci (112–150 × 6–7.5 μm), and equilaterally ellipsoid ascospores.

Discussion

In this paper, five new species of *Hymenoscyphus* are described based on morphological characters that are congruent with phylogenetic inference. Our study revealed that combining morphological with molecular data is important for defining a species. In the case of *H. aurantiacus*, the stipe surface morphology differs somewhat between the two collections as mentioned previously, yet the sequence identity and major morphological similarities support them as conspecific. The minor stipe surface differences among collections may be considered infraspecific variations.

The previous phylogenetic studies of *Hymenoscyphus* were preliminarily ITS-based using small sample sizes (Zhang & Zhuang 2004, Baral et al. 2006, Han & Shin 2008, Johnston & Park 2013, Zheng & Zhuang 2013a). Our analyses have been based on more extensive sampling and multi-gene sequence data, which provide a more comprehensive understanding of the genus. Our phylogenetic reconstruction produced six main clades within *Hymenoscyphus*. *Hymenoscyphus fructigenus*, the type species of the genus, is located in Clade A together with *H. albidoides*, *H. albidus*, *H. caudatus*, *H. dehlii*, *H. fucatus*, *H. macroguttatus*, *H. fraxineus*, *H. scutuloides*, *H. serotinus*, and *H. sinicus* in addition to four new taxa described here (*H. macrodiscus*, *H. qinghaiensis*, *H. tetrasporus, H. yui*). These fungi represent the core group of *Hymenoscyphus.* Generally, species in this clade have relatively large apothecia, rectangular cells in ectal excipulum, and scutuloid ascospores.

The seven species—*H. brevicellulus, H. ginkgonis*, *H. hyaloexcipulus, H. microcaudatus, H. microserotinus, H. subpallescens, H. subsymmetricus*—that comprise Clade B possess rectangular ectal excipular cells and scutuloid or ellipsoid ascospores usually less than 20 µm long. Clade C contains *H. crataegi*, *H. globus,* and *H. immutabilis,* which share the presence of angular, isodiametric to subglobose cells in the ectal excipulum and fusoid, symmetrical ascospores less than 15 µm long. The three taxa in Clade D share yellowish apothecia and the presence of croziers at ascus base.

Hymenoscyphus berggrenii, *H. haasticus, H. kiko*, *H. ohakune,* and *H. waikaia*, species so far known only from New Zealand (Johnston & Park 2013), comprise Clade E. They are similar in that the paraphyses branch on their upper portions to form an epithecium-like layer across the top of the asci and by occurring on *Nothofagus* leaves. The phylogenetic position of these fungi may be influenced by geographic separation and host restriction.

Hymenoscyphus aurantiacus and *H. calyculus,* which form Clade F, resemble each other in their yellow to orange apothecia and J+ asci with croziers at base. They appear to be similar to taxa in Clade D but are differentiated by their sequence data.

Analyses of individual gene sequences showed that ITS and BTU are efficient in distinguishing the species investigated, while LSU is problematic in some cases due to its limited interspecific nucleotide divergence. Based on the information gathered, it is still difficult to fully understand the phylogeny of *Hymenoscyphus*. Evolutionary relationships among species and clades need further exploration by integrated studies. Along with increasing the number of taxa and features investigated within the genus, a better and more reasonable phylogeny of *Hymenoscyphus* will be displayed.

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